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Vibrio cholerae cytolysin: assembly and membrane insertion of the oligomeric pore are tightly linked and are not detectably restricted by membrane fluidity

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Abstract

Hemolytic strains of *Vibrio cholerae* secrete a cytolysin that, upon binding as a monomer, forms pentameric pores in animal cell membranes. Pore formation is inhibited at low temperature and in the absence of cholesterol. We here posed the following questions: firstly, can oligomerization be observed in the absence of pore formation? Secondly, is membrane fluidity responsible for the effect of temperature or of cholesterol upon pore formation? The first issue was approached by chemical cross-linking, by electrophoretic heteromer analysis, and by electron microscopy. None of these methods yielded any evidence of a non-lytic pre-pore oligomer. The second question was addressed by the use of two susceptible liposome models, consisting of cholesterol admixed to bovine brain lipids and to asolectin, respectively. The two liposome species clearly differed in membrane fluidity as judged by diphenylhexatriene fluorescence polarization. Nevertheless, their permeabilization by the cytolysin decreased with temperature in a closely parallel fashion, virtually vanishing at 5°C. Omission of cholesterol from the liposomes uniformly led to an increase in membrane fluidity but prevented permeabilization by the cytolysin. The effects of temperature and of cholesterol upon cytolysin activity are thus not mediated by fluidization of the target membrane. The findings of our study distinguish *V. cholerae* cytolysin from several previously characterized pore-forming toxins.

Keywords: Pore forming toxin; Protein oligomerization; Membrane fluidity

1. Introduction

Subsequent to the discovery that *Staphylococcus aureus* α-hemolysin forms oligomeric pores in the membranes of animal cells [1], the same mode of action has been assigned to a steadily growing number of bacterial toxins. Typically, the first step toward pore formation consists in membrane binding of monomeric toxin molecules, whereas permeabilization requires assembly of the bound monomers into oligomers of defined stoichiometry that assume a bilayer-inserted conformation. In contrast to binding which readily occurs at both high and low temperatures, permeabilization is inhibited at low tem-

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perature. With various toxins, the latter restriction has been attributed to decreased fluidity of the target lipid bilayer [2–4]. When prevented from membrane insertion, the bound toxin molecules may accumulate as oligomeric 'pre-pores' [5–8]. The occurrence of pre-pores supports the view that, in the process of pore-formation, oligomerization precedes membrane permeabilization. In the present study, we asked if the two widespread principles of pre-pore assembly and of restriction of activity by low membrane fluidity also apply to *Vibrio cholerae* cytolysin (VCC). The lytic activity of VCC not only depends on an elevated temperature but also requires both cholesterol [9] and ceramides [10] in the target membrane, although even in their absence the monomer binds to bilayers [11] or immobilized lipids [12]. This permitted us to examine the bound monomeric toxin for the assembly of pre-pores under a variety of conditions not compatible with membrane permeabilization. The cytolysin was either incubated with liposomes containing all necessary lipids but at low temperature, or it was allowed to react at permissive temperature but with membranes lacking one or the other essential lipid. Within this varied set of samples, oligomeric pre-pores were sought using an array of biochemical techniques. For all these efforts, no indication of pre-pore formation was obtained. Thus, in contrast to other bacterial pore-forming toxins, oligomerization apparently cannot be dissociated from membrane penetration. The permeabilization of various target liposomes by the cytolysin was quantitated by means of fluorescent marker release. Permeabilization was then correlated with membrane fluidity which was independently examined by diphenylhexatriene fluorescence polarization. Unexpectedly, the experimental findings did not support a role of membrane fluidity in the activity of VCC. The collective results indicate that VCC deviates from the molecular mechanism of pore-formation that has been outlined previously with 'prototypic' toxins such as staphylococcal α-hemolysin.

2. Materials and methods

2.1. Bacterial strains

V. cholerae O1 El Tor strain 8731, which was used for isolation of the VCC 65 kDa form, was a generous gift of Dr R. Hall, Washington, DC. The 50 kDa form was isolated from *V. cholerae* O1 El Tor strain KM 169 [13].

2.2. Purification of V. cholerae cytolysin

Both forms of VCC were isolated according to published procedures. Briefly, the 65 kDa form was precipitated from culture supernatants of strain 8731 with ethanol and purified by sequential isoelectric focusing and hydroxyapatite chromatography [11]. For the isolation of the 50 kDa form, *V. cholerae* strain KM 169 was cultured in liquid minimal medium. The cytolysin was purified by ammonium sulfate fractionation and sequential chromatography on DE-52 cellulose (Whatman), Ultrogel AcA-44 (LKB) and a Mono Q column (Pharmacia) [13].

2.3. Preparation of liposomes

Egg yolk phosphatidylcholine, galactosylceramide, cholesterol, bovine brain extract (a mixture of phospho- and glycolipids with ca. 1% residual cholesterol) and asolectin (a crude lipid extract from soy bean) were purchased from Fluka, Buchs, Switzerland, dissolved in chloroform and stored at −20°C. The lipids were admixed in the ratios indicated in Section 3 and dried down from the solvent under a stream of nitrogen in a round bottom flask. The resulting glassy lipid film was dried for 30 min under vacuum. Following suspension of the lipids to 5 mg/ml in 20 mM Hepes/150 mM NaCl, large unilamellar vesicles (LUV) were formed by repeated extrusion of the suspension through polycarbonate membranes (Corning/Whatman; 100 nm pore size) according to [14], whereby the extrusion apparatus (Lipex Biomembranes, Vancouver, Canada) was thermostated at 45°C when processing brain lipids. The lipid concentration in the final sample was determined using a commercial enzymatic cholesterol assay (Boehringer Mannheim) and by phosphorus analysis according to [15]. In one experiment, liposomes prepared from brain lipids were subsequently enriched with cholesterol solubilized with methyl-β-cyclodextrin (Sigma, Deisenhofen, Germany) according to [16].

2.4. Isolation of alkaline-stable glycolipids from crude soybean lipid extract (asolectin)

The occurrence of a glycosphingolipid constituent within the commercial asolectin was confirmed by thin layer chromatography on silica gel 60 HPTLC plates (Art. 5633, Merck, Darmstadt, Germany), developed with chloroform/methanol/water (120:70:17, by volume), whereby the glycolipids were visualized by reaction with α-naphthol [17] and with orcinol [18], and glucosylcerebroside from human spleen (Sigma) served as a standard. For preparative purposes, asolectin (0.35 g) was suspended in 25 ml aqueous NaOH (1 M) and incubated for 1 h at 37°C. The sample was then neutralized with acetic acid, dialyzed, lyophilized, and finally dissolved in 5 ml of chloroform. One ml of the solution was applied to preparative TLC. The glycolipid bands were detected on a UV screen using primuline [17,19], scraped off and extracted with chloroform/methanol/water (30:60:8, by volume).

2.5. Calcein release assay

Large unilamellar vesicles (LUV) were produced as above, whereby the lipids after drying were resuspended in Hepes/NaCl containing 50 mM calcein (2′,7′-bis-[*N*,*N*-bis-(carboxymethyl)-aminomethyl]-fluorescein). Following extrusion, the liposome suspension was passed over a column of Sephadex G50 (Pharmacia) equilibrated with Hepes/NaCl to remove non-entrapped calcein. The void volume fractions were pooled, and the lipid concentration was determined. Liposomes (15 μg of lipid in a final volume of 100 μl) were incubated for 10 min at 37°C with the amounts of cytolysin indicated in Section 3. Subsequently, each sample was diluted into 3 ml of Hepes/NaCl (pH 7.5) and immediately assayed for calcein fluorescence (λ_{ex} 488 nm, λ_{em} 520 nm) in a SPEX Fluoromax spectrofluorimeter. The fraction of calcein released was calculated from the increase of fluorescence over that of a control sample incubated without the cytolysin, whereby the fluorescence maximum corresponding to 100% release was determined on a sample solubilized with sodium deoxycholate (final concentration 6 mM).

For kinetic measurements, liposomes containing 15 μg total lipid were diluted into 3 ml Hepes/NaCl with 0.05% bovine serum albumin. The samples were stirred inside the thermostated spectrofluorimeter and allowed 2 min for thermal equilibration. The experiments were then started by addition of VCC (5 μg), and the calcein fluorescence was followed for 5 min.

2.6. Measurements of diphenylhexatriene fluorescence anisotropy

To 2 μg of diphenylhexatriene (DPH) dissolved in 5 μl dioxane, a suspension of the membrane preparation in question (0.8 mg of lipid in 150 μl Hepes/NaCl) was added. Following incubation for 30 min in the dark, the mixture was diluted to 3 ml and transferred into the thermostated fluorimeter now equipped with polarizers in the excitation and the emission light paths. From the fluorescence intensities (λ_{ex} 350 nm, λ_{em} 452 nm) measured at both parallel and perpendicular orientations of the polarizers, the fluorescence anisotropy was calculated according to [20].

2.7. Polyacrylamide gel electrophoresis (PAGE) of VCC oligomers

VCC (20 μg/ml) was added to the liposome preparation in question (total lipid, 100 μg/ml) and incubated for 30 min at 37°C. The samples were then solubilized at room temperature by addition of sodium dodecyl sulfate (SDS; 0.5% final concentration) and subjected to SDS–PAGE. For native PAGE, gels were prepared with 5% acrylamide, supplemented with 1,4 bis(acryloyl)piperazine (Fluka) as the cross-linker (molar ratio to acrylamide 0.015). The samples were solubilized with deoxycholate. Electrophoresis was carried out in the presence of the latter detergent (6 mM) and of Tris and glycine (50 mM each) at 5 V/cm for 1.5 h. Protein bands were visualized by silver staining [21] or with Coomassie blue R250.

2.8. Chemical cross-linking of oligomeric VCC

To allow for oligomerization, VCC (final concentration, 0.2 mg/ml) was first incubated with the liposome species in question (total lipids, 2.5 mg/ml) in 50 mM Hepes (pH 8.0) at 37°C or at 4°C as indi-

cated in Section 3. The cross-linking reagent disuccinimidyl suberate (DSS, Pierce; dissolved to 10 mM in dimethylformamide) was then added to 60 μM, and the sample was incubated again. The reaction was stopped by addition of Tris (pH 8.0) to 100 mM. The samples were then supplemented with SDS to 1%, heated to 95°C for 5 min, and analyzed by SDS–PAGE. In a second set of experiments, difluorodinitrobenzene (DFDNB, Fluka) was used instead of DSS, with the modifications that the reaction was buffered at pH 7.0 and that DFDNB was employed at 2 mM final concentration.

2.9. Electron microscopy

Negatively stained specimens were prepared on glow discharge-treated continuous carbon support films by the droplet negative staining procedure [22]. Liposome–VCC solutions (with a lipid concentration of 5 mg/ml and VCC concentration of 0.1 mg/ml) were adsorbed to the carbon films, washed three times with distilled water to remove buffer salts and then negatively stained with 5% ammonium molybdate (pH 7.0) containing 1% trehalose [23]. Room temperature study of air-dried negatively stained specimens was performed using a Zeiss EM900 transmission electron microscope, under conventional electron dose irradiation conditions at minimum brightness setting. Electron micrographs were routinely recorded at image magnifications between ×30 000 and ×85 000, on Kodak type 4489 electron image film.

3. Results

3.1. Asolectin supplemented with cholesterol provides a sensitive membrane model for the study of VCC pore formation

Fig. 1A displays the cytolysin-mediated release of calcein from liposomes consisting of asolectin (an extract of lipids from soy bean), either with or without cholesterol supplementation. For comparison, results are also shown for vesicles produced with egg yolk phosphatidylcholine instead of asolectin. Evidently, the asolectin/cholesterol liposomes amply exceeded the others in sensitivity to VCC. As expected,

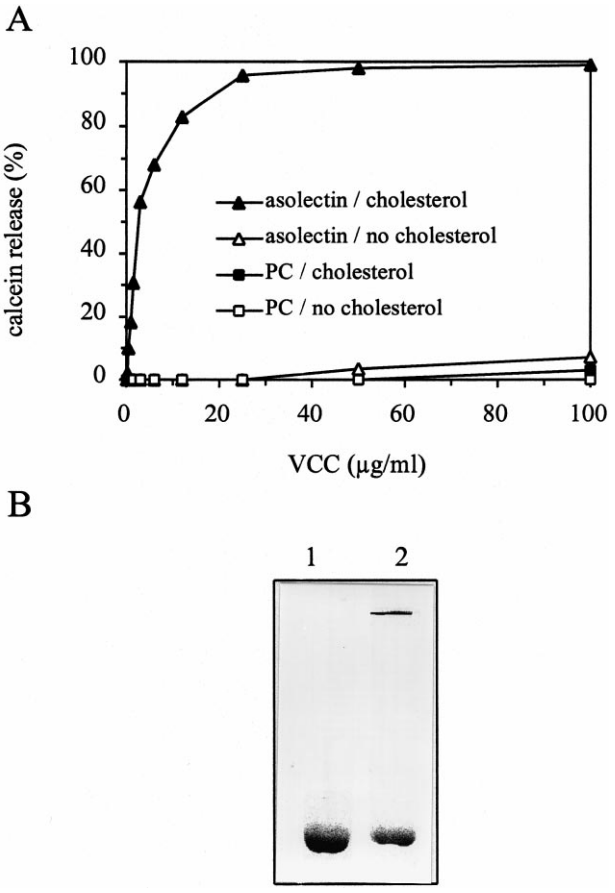

Fig. 1. Permeabilization of asolectin/cholesterol liposomes by *Vibrio cholerae* cytolysin (VCC). (A) Calcein release. Liposomes with entrapped calcein were produced from asolectin or phosphatidylcholine, without or with addition of cholesterol (20% by weight). To samples containing 150 μg/ml of lipid, VCC was added at the amounts indicated. Following incubation at 37°C for 10 min, the fraction of calcein released into the supernatant was fluorimetrically determined. (B) Oligomerization of VCC (SDS–PAGE). 1, monomeric VCC (2 μg); 2, identical amount of VCC following incubation with asolectin/cholesterol liposomes(10 μg of lipid). The ensuing upper band corresponds to the pore pentamer.

membrane permeabilization correlated with formation of the SDS-resistant VCC pentamer (Fig. 1B).

In a previous study employing model membranes prepared from bovine brain lipids, it was found that, apart from cholesterol [9], VCC pore formation also critically required the simultaneous presence of ceramides, whereby free ceramide and monoglycosylceramides proved similarly effective [10]. The asolectin employed in this study also contained a lipid species most probably representing a glycosphingolipid (see Section 2) and which, when purified by

TLC, conferred susceptibility to the cytolysin upon liposomes otherwise consisting of phosphatidylcholine and cholesterol. These findings suggest that the sensitivity of the asolectin membranes (supplemented with cholesterol) accrues from their content of a glycosphingolipid [24].

3.2. *Asolectin and bovine brain lipids yield liposomes differing in membrane fluidity*

As a prerequisite for studying the influence of membrane fluidity upon VCC pore formation, we next characterized our model liposomes at different temperatures using the lipophilic fluorescent probe diphenylhexatriene (DPH). This molecule partitions into membranes where it aligns with the acyl side chains of the lipid molecules. Consequently, its motional freedom reflects that of the surrounding lipids, and it is inversely related to the anisotropy of its fluorescence, which thus provides an indicator of membrane fluidity [25]. For control purposes, we first studied membranes prepared from dimyristoylphosphatidylcholine (DMPC). Fig. 2A shows that, with liposomes consisting of DMPC only, the DPH anisotropy was high up to 24°C but steeply declined when the temperature was raised above that value. This indicates transition of the DMPC membranes from the gel into the liquid crystalline state and is in good agreement with previous calorimetric and spectroscopic data [26,27]. Conversely, when liposomes were prepared from DMPC with cholesterol (20% by weight), the fluorescence polarization decreased only slightly and evenly with rising temperature. Again in keeping with previous findings, this indicates that cholesterol abolishes the clear-cut phase transition of the DMPC bilayer, which is thus rendered less fluid at elevated temperature. With brain lipid membranes, a phase transition was observed at 30°C (Fig. 2B) and was again prevented by addition of cholesterol. Conversely, asolectin membranes exhibited a distinctly lower DPH anisotropy throughout the entire temperature range; this

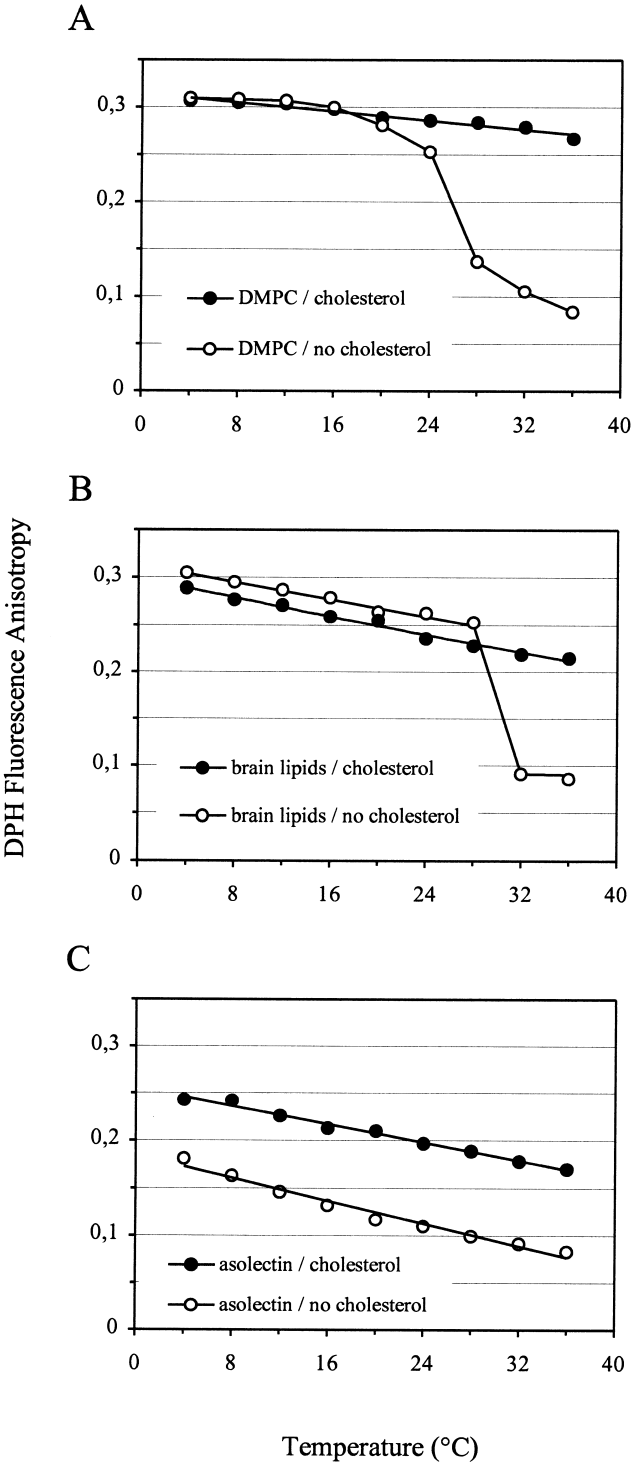

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Fig. 2. Characterization of liposome membrane fluidity by diphenylhexatriene (DPH) fluorescence polarization. Liposomes were prepared from dimyristoylphosphatidylcholine (DMPC; A), bovine brain lipids (B), or asolectin (C), either with or without addition of cholesterol (20% by weight). The liposomes were spiked with DPH (final ratio DPH/lipid, 1:400 by weight), and the fluorescence anisotropy of the probe was measured at varying temperatures. A drop in fluorescence anisotropy corresponds to an increase in membrane fluidity.

tallies with the known fluidizing effect of unsaturated acyl chains that are more prevalent in plant than in mammalian phospholipids. Once more, fluorescence anisotropy was enhanced by supplementation with 20% cholesterol. The plot of anisotropy vs. temperature (Fig. 2C) parallels that obtained with brain lipid/cholesterol liposomes (Fig. 2B). However, while with the latter lipids the anisotropy is 0.25 at 20°C, the same value is attained with asolectin/cholesterol bilayers at 4°C. It may thus be estimated that, with the two membranes in question, states of similar fluidity are separated by a temperature interval of about 15°C.

In the original work on the use of DPH as a probe membrane fluidity [25,28], the interior of the lipid bilayer was treated as an isotropic environment, characterized by a uniform viscosity that in turn would determine fluidity. The relationship between membrane viscosity and the fluorescence anisotropy of DPH can then be expressed as follows:

$$\eta = \frac{k \bullet r}{r_0 - r} \quad (1)$$

where η is the viscosity and k comprises several parameters characteristic of the fluorophore; r is the experimental anisotropy, whereas r_0 is the fundamental anisotropy of DPH which has been determined to be 0.36 [25]. By application of Eq. 1 to the experimental anisotropies at 5°C and at 35°C, respectively, it would follow that within this temperature interval the viscosity of the membrane changes by a factor of about 2.5 (brain lipids/cholesterol) or 2 (asolectin/cholesterol).

3.3. The influence of temperature upon VCC pore formation is not mediated by membrane fluidity

Bacterial pore-forming cytolysins typically bind to membranes at both high and low temperature, but

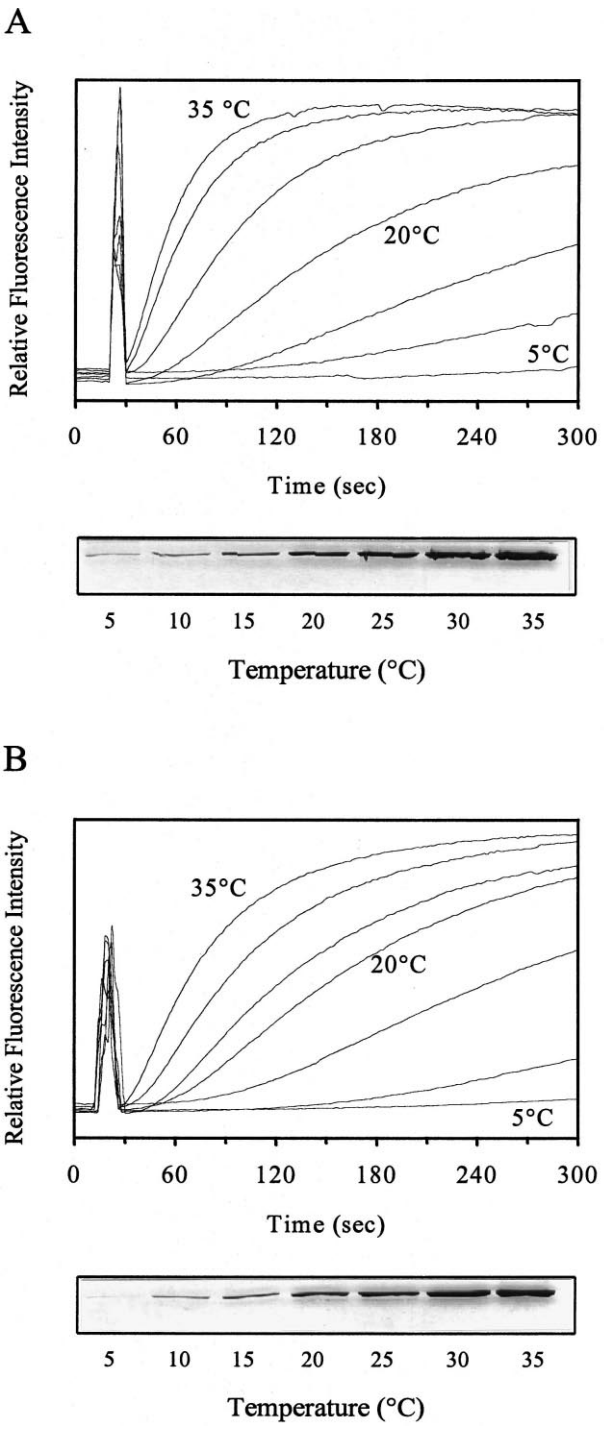

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Fig. 3. Temperature dependency of pore formation. Liposomes (15 μg lipid) with entrapped calcein and consisting of brain lipids/cholesterol (A) or asolectin/cholesterol (B) were stirred inside a fluorimeter that was thermostated at temperatures ranging from 5°C to 35°C (interval: 5°C). Thirty seconds after starting the measurements, VCC (5 μg) was added, and the calcein fluorescence was followed for 5 min. An increase of fluorescence intensity indicates release of calcein from permeabilized liposomes. Insets: SDS–PAGE showing VCC oligomers formed on the same liposomes and at the same temperatures as above.

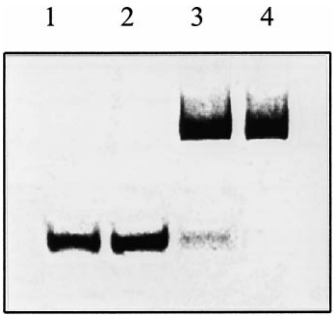

Fig. 4. Non-denaturing PAGE of VCC monomers and oligomers. Sodium deoxycholate was used for sample solubilization and electrophoresis. Lane 1, monomeric VCC; lane 2, VCC incubated with brain lipid/cholesterol liposomes at 4°C; lane 3, VCC incubated with brain lipid/cholesterol liposomes at 37°C; lane 4, oligomeric VCC purified by gel filtration. Oligomerization is not apparent on liposomes at 4°C.

membrane permeabilization is often diminished or even abrogated at low temperature. Under the latter condition, the motional freedom of both the membrane lipids and the cytolysin molecule will be restricted, and either effect might conceivably account for the inhibition of pore formation. If membrane fluidity was responsible, the temperature dependency of pore formation should vary with the target membrane. One would thus expect that, when gradually shifting the temperature from high to lower values, the activity of *V. cholerae* cytolysin would diminish and disappear more readily with the more rigid brain lipid/cholesterol membranes as compared with the more fluid ones prepared with asolectin. This, however, was not the case: when the temperature was decreased stepwise from 35°C to 5°C, the rate of calcein release from liposomes of either type was slowed down in a closely parallel fashion and in both instances virtually vanished at 5°C (Fig. 3). We conclude that the fluidity of the respective target membranes is not crucial to the activity of VCC, and that the observed variation in the rate of pore formation is due to the effect of temperature upon the cytolysin molecule itself.

3.4. *Absence of oligomeric 'pre-pores' of VCC under conditions that prevent membrane permeabilization*

In the formation of oligomeric pores from membrane-bound toxin monomers, the lateral aggregation of the oligomer subunits may be conceptually distinguished from their insertion into the lipid bilayer. Then, with VCC, either of these two events might be subject to the inhibition imposed by low temperature or by the lack of cholesterol or ceramide in the membrane. If insertion was selectively affected by one or the other of these inhibitory conditions, the bound toxin should accumulate as non-lytic, oligomeric 'pre-pores'. To test this hypothesis, we again used liposomes of different compositions. Liposomes essentially insensitive to VCC pore formation [10] were prepared from phosphatidylcholine (100%), from phosphatidylcholine with cholesterol (20% by weight), from phosphatidylcholine with ceramide (40%), and from bovine brain lipids without cholesterol supplementation. The insensitive liposomes were incubated with the cytolysin at 37°C. Sensitive liposomes were prepared from phosphatidylcholine with ceramide (40%) and cholesterol (20%), and by cholesterol supplementation (20%) of bovine brain lipids. The sensitive liposomes were exposed to the cytolysin at 0°C and, for control purposes, also at 37°C. On all the samples, various procedures were

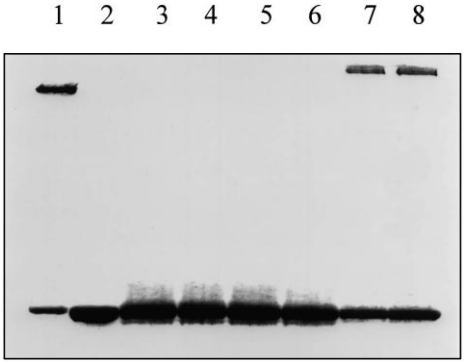

Fig. 5. Chemical cross-linking of VCC oligomers with disuccinimidyl suberate. VCC was incubated with various liposome species at 4°C (30 min) or 37°C (15 min) to allow for oligomerization. They were then exposed to disuccinimidyl suberate at 4°C (30 min) or 37°C (15 min) in order to covalently cross-link the oligomers. The samples were heated to 95°C with SDS and analyzed by SDS–PAGE. The lipid composition and incubation conditions of the samples displayed in lanes 1–8 of this figure are given in Table 1. Cross-linking readily proceeds both at 37°C (lane 7) and at 4°C (lane 8); due to their denatured state, the cross-linked oligomers migrate slower than the native ones (cf. lane 1) which are readily dissociated by heat (lane 2). Lanes 3–6 show that, at 4°C or in the absence of either cholesterol or galactosylceramide, no oligomers are detectable by cross-linking.

then performed in order to detect formation of pre-pores. In contrast to several functionally related toxins [7,8], pre-pores of VCC are not visualized by SDS–PAGE (cf. Figs. 1B and 3). Therefore, our first attempt was to replace SDS by the mild detergent deoxycholate during sample solubilization and electrophoresis. This electrophoretic system readily resolved the monomer from the pore pentamer. However, no pentamers were detected on sensitive membranes at 4°C (Fig. 4) or with any of the insensitive membranes at 37°C (not shown).

While less denaturing than SDS, deoxycholate might still be disruptive to non-covalently bonded oligomers. Therefore, following incubation of liposomes with the cytolysin, we tried to stabilize the hypothetic pre-pores with the cross-linking reagents disuccinimidyl suberate or difluorodinitrobenzene. The samples were then subjected to SDS–PAGE. Once again, oligomers were detected neither in the absence of cholesterol or ceramide nor at 4°C on sensitive membranes (Fig. 5 and Table 1). However, one might argue that in the above experiments the pre-pores could selectively have escaped from cross-linking due to a more loosely bonded or otherwise unfavorable conformation that would prevent reactive side chains of neighboring monomers from suitably aligning. We thus devised a strategy to detect pre-pore formation at low temperature that would rely on neither resistance to detergents nor on steric compliance with chemical cross-linking. Two naturally occurring proteolytic variants of the cytolysin were employed with molecular masses of 50 and 65 kDa, respectively. These molecules can form both homogeneous and hybrid pentamers which are readily resolved on SDS–PAGE [10]. Brain lipid/cholesterol liposomes were first exposed to the 65 kDa variant, passed over Sepharose 6B to remove unbound cytolysin, and incubated; all these steps were carried out at 4°C. The 50 kDa form was then added, and the temperature was raised to 37°C. The rationale of this experiment was the following: if the toxin could assemble into oligomeric pre-pores on membranes at low temperature, this process would lead to consumption of the 65 kDa form prior to the addition of the smaller variant. The latter then could only form pre-pores on its own. The

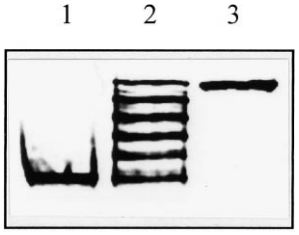

Fig. 6. Oligomers generated from two forms of VCC differing in molecular mass (SDS–PAGE). Lane 1, oligomers of the 50 kDa variant; lane 3, oligomers of the 65 kDa variant. Oligomers were formed by incubation of the toxins with brain lipid/cholesterol liposomes at 37°C. Lane 2, Liposomes were first incubated with the 65 kDa form at 4°C for 15 min. After addition of the 50 kDa form, the temperature was raised to 37°C for 15 min prior to electrophoretic analysis. The preferential formation of hybrid oligomers indicates that the 65 kDa form had not been consumed by the formation of oligomeric 'pre-pores' during the first incubation at 4°C (see text).

Table 1
Composition of liposomes and conditions for oligomerization and chemical cross-linking of oligomers employed in the experiment depicted in Fig. 5.

Sample (lane)	Liposome composition	Oligomerization at temperature	Cross-linking (at temperature)	Heating before PAGE
1	Brain lipid/cholesterol (20%)	37°C	No	No
2	Brain lipid/cholesterol (20%)	37°C	No	Yes
3	Phosphatidylcholine	37°C	37°C	Yes
4	Phosphatidylcholine/galactosylceramide (40%)	37°C	37°C	Yes
5	Phosphatidylcholine/cholesterol (20%)	37°C	37°C	Yes
6	Phosphatidylcholine/galactosylceramide (40%)/ cholesterol (20%)	4°C	4°C	Yes
7	Phosphatidylcholine/galactosylceramide (40%)/ cholesterol (20%)	37°C	37°C	Yes
8	Phosphatidylcholine/galactosylceramide (40%)/ cholesterol (20%)	37°C	4°C	Yes

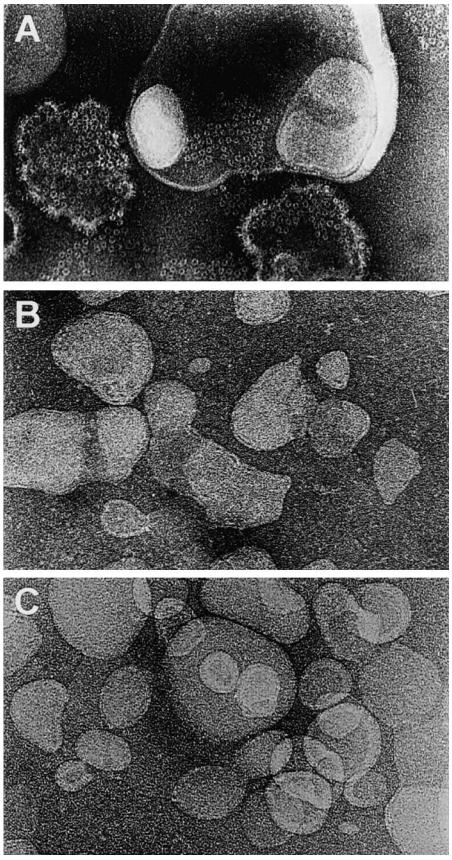

Fig. 7. Electron microscopy of VCC oligomers. (A) Liposomes of phosphatidylcholine+40% ceramide and 20% cholesterol, following incubation with VCC. Ring-shaped oligomers have formed and are seen both attached to the liposomes and freely dispersed on the background, having been released from the liposomes. (B) Phosphatidylcholine liposomes containing ceramide but no cholesterol. (C) Phosphatidylcholine liposomes containing cholesterol but no ceramide. With both these samples, addition of VCC did not produce oligomers. All specimens were negatively stained with 5% ammonium molybdate containing 1% trehalose (pH 7.0), following adsorption to a carbon support film.

increase in temperature would unleash membrane penetration of the two pre-pore species, which concomitantly would gain resistance to dissociation by SDS. Consequently, on SDS–PAGE, the two homogeneous pentamer species should prevail, but little hybrid oligomers should be observed. In contrast, if no pre-pores would form at low temperature, oligomerization would be deferred to the incubation at 37°C. Then, from the two monomeric species already present, hybrid oligomers should readily form and be detected by SDS–PAGE. Fig. 6 shows that, at the end of this experiment, hybrid oligomers were indeed prevalent. We conclude that pre-pores of VCC had not formed at 4°C. The same experimental approach was also applied to brain lipid liposomes that were incubated with the 65 kDa cytolysin at 37°C but were lacking cholesterol at this stage. Following the addition of the 50 kDa variant, the liposomes were enriched with cholesterol using methyl-β-cyclodextrin and incubated again at 37°C prior to solubilization and SDS–PAGE. Once more, hybrid pentamers prevailed (data not shown), indicating that pre-pores had not formed in the absence of cholesterol.

To confirm the results of the above electrophoretic experiments, liposome samples incubated with VCC were also examined by electron microscopy. Liposomes consisting of phosphatidylcholine with 40% ceramide and 20% cholesterol elicited formation of numerous VCC oligomers (Fig. 7A), resembling those previously demonstrated on erythrocyte membranes [9]. Oligomers were also observed with liposomes prepared from bovine brain lipids with 20% cholesterol (not shown). In contrast, when liposomes were employed that were lacking either cholesterol (Fig. 7B) or ceramides (Fig. 7C), no VCC oligomers were detected. Unsurprisingly, they were also lacking from liposomes consisting of phosphatidylcholine only (not shown). In conclusion, both electrophoretic and morphological means did not yield any evidence of oligomeric pre-pores forming under conditions that restrict membrane permeabilization.

4. Discussion

With only few of the many pore forming toxins known have the processes of oligomerization and membrane-insertion been analyzed in molecular detail. So far, these studies have elucidated two different molecular mechanisms of pore-formation that are represented by *S. aureus* α-hemolysin and by streptolysin O. A distinguishing feature of these two mechanisms lies in their respective sequence of oligomer assembly and membrane insertion. In the case of α-hemolysin, oligomeric assembly precedes insertion [5,29], whereas they proceed in parallel with the much larger streptolysin O pore [30]. One of the aims of the present study was to define the sequence of events applicable to VCC. While this toxin shares

with streptolysin O a requirement of membrane cholesterol [9], it closely resembles α-hemolysin in pore size (1–1.5 nm; [31–33]). Our failure to observe prepores of VCC does not imply that they do not exist. However, a reasonable conclusion from the present findings is that oligomer assembly and membrane insertion are more tightly linked than in case of α-hemolysin. As stated above, oligomerization and insertion proceed concurrently with streptolysin O, which gives rise to intermediate oligomers exhibiting stoichiometries in between the monomer and the fully circularized oligomer [30,34]. No such partial oligomers have been detected with VCC, which therefore resembles α-hemolysin in this respect. Taken together, the available data suggest that oligomerization of VCC may still precede membrane insertion but that in the overall process the pre-pore does not represent a meta-stable stage.

The failure of VCC to form pre-pores on membranes lacking either cholesterol or ceramide indicates that both lipids are required not only in membrane insertion but very early in the mutual interaction of the membrane-bound monomers leading to oligomer assembly. A similar role for cholesterol has recently been demonstrated with the streptolysin O monomer, which requires allosteric activation by the sterol to enable oligomerization and pore formation [35]. With both streptolysin O [36] and VCC [9], several structural features of the cholesterol molecule such as the available 3β-OH group have been defined that are essential for eliciting oligomerization. It will be most interesting to determine whether the interaction of VCC with ceramide is similarly stereospecific. In contrast to oligomerization, the monomer readily binds in a non-specific fashion to bilayers that consist of no more than just phosphatidylcholine [11]; therefore, in a natural environment, the toxin may conceivably be distracted from its mammalian target membrane by adsorption to different lipid surfaces. However, since binding is also reversible [11], the bound cytolysin is not wasted prior to oligomerization. Thus, in a sense, the early requirement of cholesterol and ceramides in oligomerization may compensate for the lack in specificity of membrane binding.

The second topic of the present study was the role of membrane fluidity in formation of the VCC pore. The impact of membrane fluidity is often studied with pure phosphatidylcholine vesicles. With chemically defined PC membranes, small shifts of temperature may elicit sharp transitions between the 'frozen' gel and the liquid crystalline states. It has been reported that only above their respective transition temperatures do liposomes prepared from various PC species permit membrane insertion of the respective α-hemolysins of *S. aureus* [4] and *Escherichia coli* [37]. These findings support a role of membrane fluidity in restricting the activity of pore-forming proteins. However, it is still an open question as to what extent this conclusion is applicable to cytoplasmic membranes of animal cells. These represent complex mixtures of lipids including cholesterol, and they therefore do not exhibit clear-cut phase transitions with varying temperature. Sharp phase transitions were also absent from the mixed-lipid liposomal membranes used in the present study. Nevertheless, the fluidity of the membranes was reduced but their susceptibility to VCC enhanced by cholesterol. This supports the view that the sterol contributes to VCC oligomerization by a specific direct interaction with the cytolysin molecule [9] rather than by favorably but non-specifically changing the physical properties of the target membrane.

The quantitative estimates of the changes in membrane fluidity with temperature should be regarded with caution, because they were derived from a simple physical model that has proven insufficient to describe the behavior of real lipid bilayers [38,39]. Nevertheless, they suggest that there is no close correlation between the fluidity of the target membrane and its susceptibility to VCC, a conclusion that is also supported by the comparison of brain lipid/cholesterol and asolectin/cholesterol membranes. If membrane fluidity does not mediate the effect of temperature upon pore formation, the most likely interpretation is that the cytolysin molecule is itself functionally affected by the temperature. In fact, assembly of toxin pores commonly involves distinct conformational transitions [40], which may conceivably require thermal activation. This hypothesis fits well with a lack of detectable 'pre-pore' oligomers at low temperature. Being more rigid in the cold, the membrane might conceivably resist insertion of the toxin molecules, but it is hard to see how it should prevent the conformational transition of superficially attached toxin monomers and their assembly into a

likewise superficially bound pre-pore oligomer. The lack of pre-pores at low temperature thus again calls for an explanation other than membrane fluidity. Finally, the results presented also make sense in the context of the lipid specificity of VCC. On cell membranes, both cholesterol and glycosphingolipids are thought to preferentially partition into so-called 'rafts' which represent microdomains also enriched with GPI-anchored proteins. These microdomains have been ascribed a particularly low degree of fluidity, a fact that is reflected by their resistance to solubilization by Triton X-100 at low temperature [41]. It thus appears that, on natural membranes, VCC has a preference for the most rigid areas, which would necessitate the ability of this toxin to resist inhibition of pore formation by low bilayer fluidity.

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